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TECHNICAL MANUSCRIPT 510

MACROPHAGE - GALACTOSIDASE AND DEMONSTRATION OF ITS ACTIVITY BY ELECTRON MICROSCOPY

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MARCH 1969

DEPARTMENT OF THE ARMY
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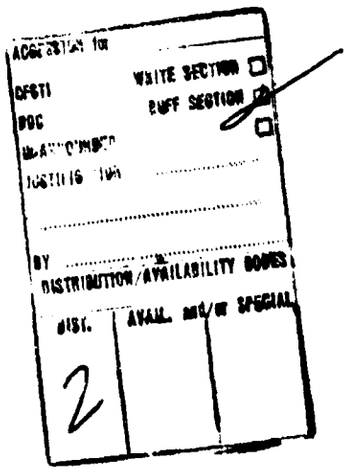

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MEDICAL SCIENCES LABORATORIES

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ABSTRACT

Hydrolases of alveolar macrophages are considered to play a role in early host resistance to airborne infection. This study biochemically examined certain properties of macrophage galactosidase and developed methods for the initial demonstration of its activity by electron microscopy. Cells were harvested from the lungs of BCG-treated rabbits and then evaluated for activity against conventional biochemical substrates and the histochemical substrate, 5-bromo-4-chloroindol-3-yl-β-D-galactopyranoside. Hydrolysis of the latter results in the formation of indigo. Enzyme partition studies of cell lysates did not reveal preferential hydrolysis, pH optima, analogue inhibition, or heat inactivation curves unique to the particulate or soluble fraction. Evaluation of processing methods for electron microscopy revealed moderate solubility of indigo in propylene oxide. Fixation of single cells in buffered glutaraldehyde provided the best compromise for preservation of cell detail and enzymatic activity. The techniques developed have been used for ultrastructural demonstration of galactosidase activity in alveolar macrophages and other inflammatory cells.

QUANTITATIVE STUDIES OF MACROPHAGE β-GALACTOSIDASE AND DEMONSTRATION OF ACTIVITY BY ELECTRON MICROSCOPY*

Hydrolases of alveolar macrophages are considered to play a role in early host resistance to airborne infection. As part of general efforts to understand host-parasite interaction, several in situ markers of enzyme activity at the tissue level have been developed in our laboratory. A recent report** examined their utility as cytochemical and semi-quantitative enzyme indicators. The present study (i) examined rabbit alveolar macrophages for certain properties of their β-galactosidase and (ii) provided quantitative data basic to this initial demonstration of galactosidase activity by electron microscopy.

Studies first were performed to characterize the enzyme by quantitative comparison of the hydrolysis of several substrates: lactose, o-nitrophenyl-β-D-galactopyranoside (ONPG), and the histochemical substrate 5-bromo-4-chloroindol-3-yl-β-D-galactopyranoside. For the latter, hydrolysis of the susceptible bond releases free indoxyls that are rapidly oxidized to form the chromogenic and substantive product, bis-indigo. The principle of this method is illustrated in Figure 1. The pH range giving maximal hydrolysis of the histochemical substrate was higher than that of the biochemical substrates which were 3.4 to 4.0 and 3.8 to 4.0 for ONPG and lactose, respectively (Fig. 2). Also, Fig. 2A shows that maximum indigo product occurs over a relatively broad range of pH values. This property is exploited to advantage in subsequent enzyme cytochemical studies. Comparison of substrate hydrolysis in different buffer systems revealed that phosphate ions caused a 30% inhibition of indoxyl galactoside hydrolysis. Similarily, inhibition was observed when slightly higher concentrations of dimethylformamide were employed than previously found necessary to prevent substrate precipitation.

Enzyme partition studies also were performed. Unfixed cell samples first were suspended in distilled water, then frozen and thawed six times. Supernatant- and particle-associated enzyme did not reveal preferential substrate hydrolysis, pH optima, or heat inactivation curves different from those of whole homogenates. Approximately two-thirds of the total activity, however, was contained in the supernatant.

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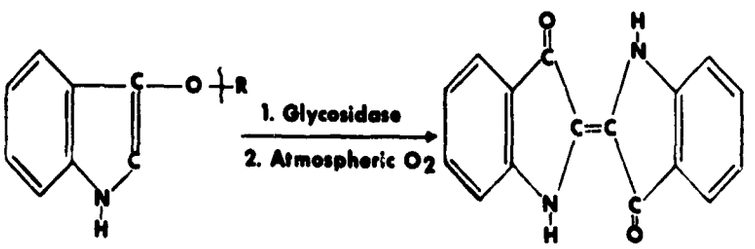
* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

** Bennett, W.E.; Pearson, B. July 1968. A study of the indigogenic principle and in vitro macrophage differentiation, (Technical Manuscript 459). Pathology Division, Fort Detrick, Frederick, Maryland.



INDOXYL GLYCOSIDE

INDIGO
(Reaction Product)

FIGURE 1. Principle of the Reaction for β-Galactosidase. The specific sugar moiety is represented at R. The indoxyl glycoside is soluble and nonchromogenic. In the presence of atmospheric oxygen, a blue-green precipitate (indigo) forms at the site of substrate hydrolysis.

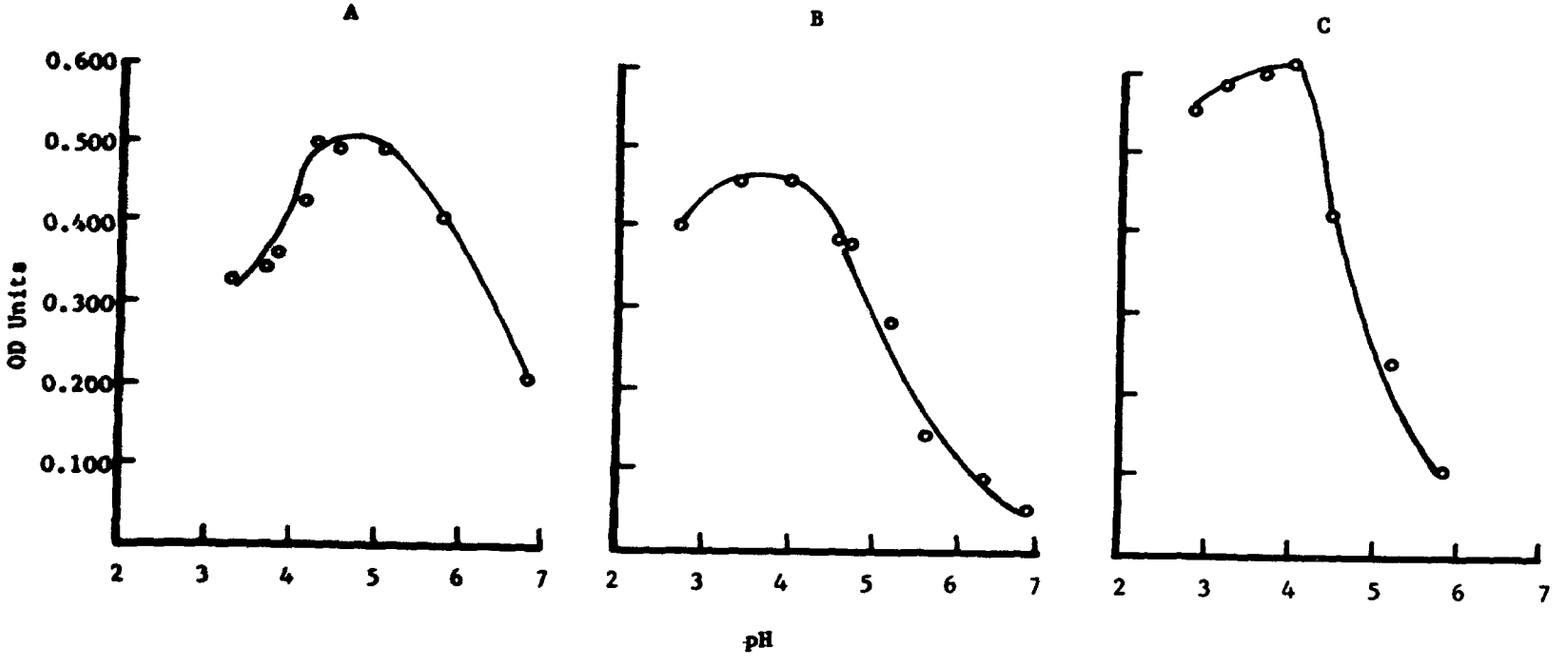


FIGURE 2. pH Activity Curves of Rabbit Alveolar Macrophage β-Galactosidase. The hydrolysis of galactosidase substrates as a function of pH: A, 5-bromo-4-chloroindol-3-yl-β-D-galactopyranoside; B, o-nitrophenyl-β-D-galactopyranoside; C, lactose.

Similar studies were conducted to examine the enzyme partition and activity of variously fixed cells. An inverse relationship exists between the duration of fixation and the preservation of cell detail on the one hand, and the activity of in situ enzyme on the other. These studies usually were performed on thick sections or carefully cut and weighed tissue blocks. Over short intervals, such samples are not exposed throughout to uniform concentrations of fixative. Accurate sampling, therefore, is difficult. The selection of alternate microtome sections for microscopy and quantitative biochemistry only partially circumvents the problem.* For this kind of analysis, the advantages of using free cells are readily apparent.

Suspensions of macrophages were fixed in glutaraldehyde (GA) or GA containing paraformaldehyde (Karnovsky's fixative) for intervals up to 1 hour, washed in buffered saline, and resuspended in 0.88 M or 0.34 M sucrose. After 30 minutes, cells were collected and resuspended, and aliquots were carefully distributed for quantitative assay. All solutions were maintained between 2 and 5 C during processing.

Fixation did not produce dramatic flattening effects on the pH activity curves for any of the substrates tested. Such flattening, however, has been observed following prolonged fixation. Karnovsky's modification occasioned a 40% greater inactivation of enzyme activity than did GA alone. Repeated freezing and thawing and addition of 0.1% final concentration Triton X-100 did not result in further activation. It was concluded, therefore, that the presence of paraformaldehyde effected destruction of enzyme rather than simply sealing of enzyme in inaccessible sites. Further studies indicated that bis-indigo formation from indoxyl galactoside hydrolysis was inhibited in the presence of Triton X-100.

Studies then were conducted to correlate GA fixation times to preservation of ultrastructural detail and cytochemical and quantitative biochemical activities. Macrophages exposed to GA for 5 minutes retained 90% of the original biochemical activity, which was completely partitioned in the sedimented fraction. Fixation for longer periods gave additional inactivation; comparison of unfixed samples with those treated for 20 or 60 minutes revealed approximately 15 and 40% inhibition, respectively. A given time of exposure to GA produced similar levels of inhibition of hydrolysis of each of the substrates. No evidence was found to support the existence of an enzyme species especially susceptible to aqueous extraction of GA inactivation.

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* Janigan, D.T. 1964. Tissue enzyme fixation studies: I. The effects of aldehyde fixation on β-glucuronidase, β-galactosidase, n-acetyl-β-glucosaminidase, and β-glucosidase in tissue blocks. Lab. Invest. 13:1038-1050.

The relatively broad pH activity curve for indoxyl galactoside hydrolysis was mentioned above. The development of weak indigo color in solutions of indoxyl galactoside substrates incubated at low pH values was most probably caused by lysosomal sensitivity to acid lysis. For this reason, the adopted cytochemical method utilized substrate at one pH unit higher than that found optimal by quantitative evaluation.

The dependence on pH of the chromogenic properties of indigo is not a factor in consideration of its electron density. Fixation, incubation time, and staining technique are the determining factors in visualizing the reaction product in the electron microscope. The cellular detail and cytochemical activity of cells processed by the methods developed to date are illustrated in Figures 3 to 6. These include 15 minutes of cold GA fixation, incubation in indoxyl galactoside for 3 hours at 37 C, postfixation in osmium, standard dehydration, and embedding, then brief staining of sections in uranyl acetate. Deposition of electron-dense reaction product was time-dependent. The specificity of the reaction was proven by inhibition of the enzyme by p-chloromercuribenzoate and galactonolactone. The product was not confined to lysosomes and was not observed over mitochondria or nuclei. Frequently, however, reaction product was observed to be associated with the nuclear envelope which also revealed ribosome-like particles at the greater magnifications.

The techniques elaborated should be applicable immediately to the ultrastructural demonstration of a variety of hydrolases for which indigo substrates have been developed. In addition, studies are planned or are in progress to correlate the present in situ findings to quantitative evaluations of isolated cell organelles.

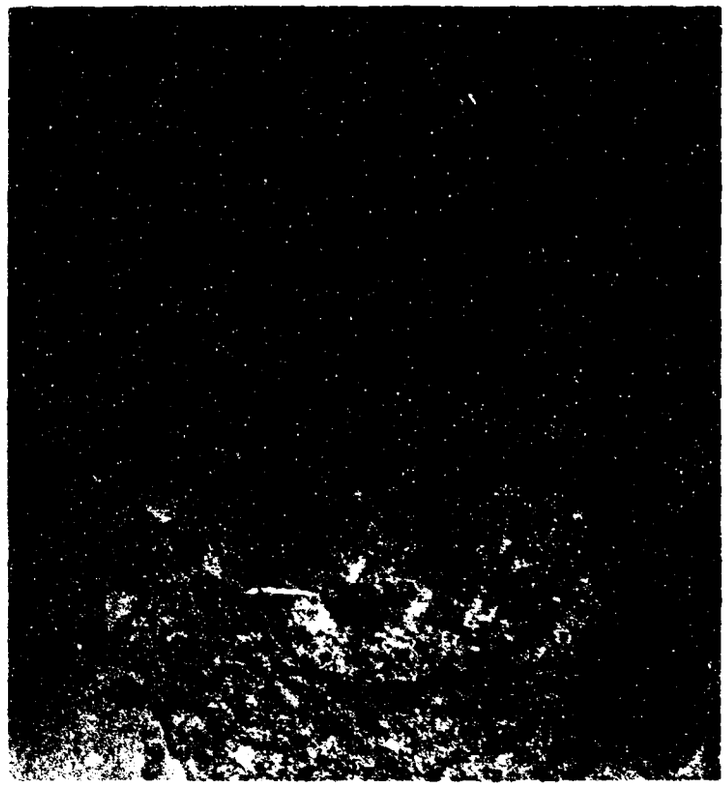

FIGURE 3. Electron Micrograph of Macrophage (7,900) Incubated for 3 Hours in Indoxyl Galactoside. Reaction product is not located over nuclei or mitochondria or attached to the limiting cell membrane. Indigo is frequently associated with dense bodies, the nuclear membrane, and cytoplasmic ground substance.

FIGURE 4. Electron Micrograph of Lymphocyte (13,400) Incubated for 3 Hours in Indoxyl Galactoside. See notes with Figure 3 caption.

FIGURE 5. Electron Micrograph of Neutrophil (13,400) Incubated for 3 Hours in Indoxyl Galactoside. See notes with Figure 3 caption.

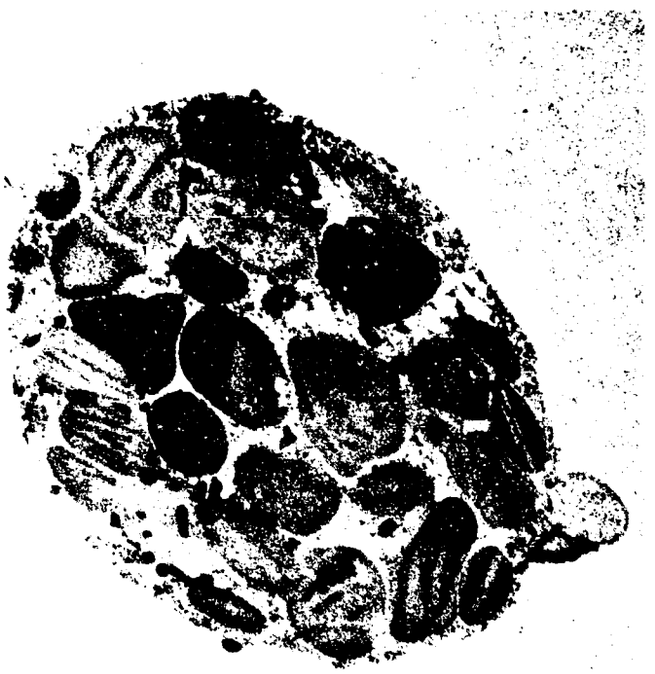

FIGURE 6. Electron Micrograph of Eosinophil (15,800) Incubated for 3 Hours in Indoxyl Galactoside. See notes with Figure 3 caption.

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14. Key Words
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